SUPEROXIDE FORMATION BY PROTOPORPHYRIN AS SEEN BY SPIN TRAPPING

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1. Introduction

The cutaneous photosensitivity of individuals with erythropoietic protoporphyria has been associated with the abnormally large amounts of protoporphyrin, found in the red blood cells [1]. Patients with erythropoietic protoporphyria are sensitive to radiation near 400 nm which corresponds with the absorption maxima of protoporphyrin [2]. The photochemical damage is thought to be the result of light absorption by the porphyrins in the skin or superficial dermal vasculature. There is still much unknown about the molecular mechanism that produces the damage. Because β-carotene ameliorates many of the symptoms, singlet oxygen has been proposed as the causative agent [2]. Moreover, a characteristic singlet oxygen product of cholesterol has been observed [3] when red blood cell ghosts are irradiated in the presence of protoporphyrin. The formation of malonaldehyde, an indicator of lipid peroxidation, with the photohemolysis of erythropoietic protoporphyric red cells has been observed [4]. However, it was observed [5,6] that only negligible amounts of malonaldehyde or the cholesterol derivative are produced during irradiation prior to leakage of hemoglobin from the red cells. Photooxidation of amino acids and photoaggregation with evidence of crosslinking of membrane polypeptides has been noted [5,7], suggesting a free radical mechanism. Thus, the question of whether a singlet oxygen or a free radical mechanism is involved in the photodamage is still unsettled.

We have observed the light-induced generation of superoxide by protoporphyrin using the technique of spin trapping. The superoxide spin adduct of 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was detected by electron spin resonance (ESR). Superoxide dismutase was able to suppress the observed signal.

2. Materials and methods

The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich Chemical Co., Milwaukee, WI. The colored impurity was removed by filtration with decolorizing charcoal using about 10 parts water to 1 part DMPO. The purified solution was frozen until used. Protoporphyrin IX was a product of Porphyrin Products, Logan, UT. Protoporphyrin (disodium salt) and superoxide dismutase were obtained from Sigma Chemical Co., St Louis, MO.

A 1 mM 'solution' (suspension) of protoporphyrin in 50 mM phosphate buffer (pH 7.0) to which DMPO was added at 50 mM was placed in an ESR spectrometer (Varian, E-4) cavity and irradiated using a projector with a 500 W tungsten bulb. The light source was placed ~15 cm from the sample. The light flux at this distance at 400 nm was measured to be 76 $\mu W/cm^2$ nm.

3. Results

The illumination of protoporphyrin in the presence of 50 mM DMPO resulted in the spectra shown in fig.1a–c. The spectrum of fig.1a. (protoporphyrin IX from Prophyrin Products) is essentially identical to that identified [9] as the superoxide spin adduct of DMPO. Figure 1b is the same sample as in fig.1a, but the scan was started ~5 min after the scan of fig.1a.

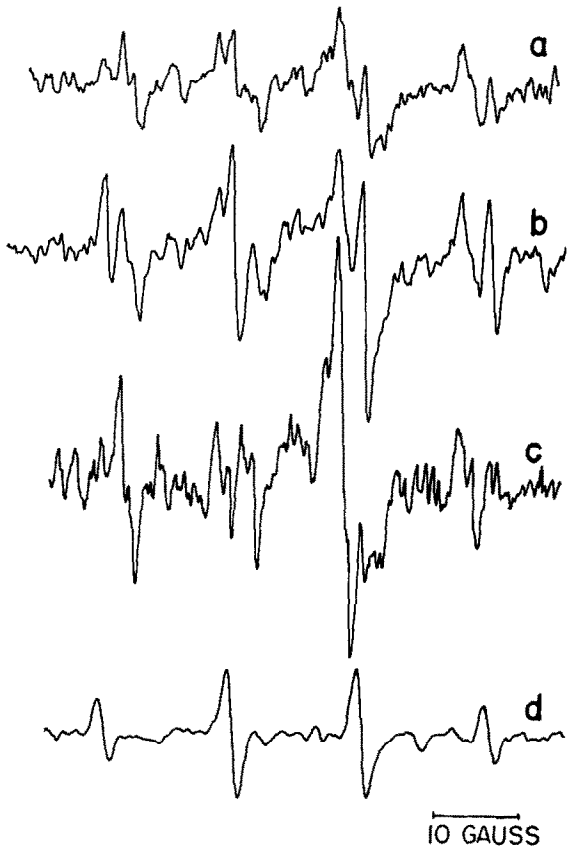

Fig.1. (a) Example of the superoxide spin adduct spectra of DMPO observed with the Prophyrin Products preparation of protoporphyrin IX. The reaction mixture contains 1 mM protoporphyrin, 50 mM DMPO in 50 mM phosphate buffer, pH 7.0. (b) The same as (a) except the scan was started 5 min later. (c) The same as (a) except the Sigma preparation of the disodium salt of protoporphyrin was used. (d) An example of the OH spin adduct of DMPO generated by a solution consisting of 50 mM DMPO, 1 mM H_2O_2 and 0.1 mM $FeSO_4$.

We no longer see just the superoxide spin adduct, but we also see significant amounts of the ·OH spin adduct. Figure 1d is an example of the ·OH spin adduct generated from Fenton's reagent. The additional signal at $g = 2.003$ in fig.1c (the Sigma preparation) is still present when the DMPO is not included in the sample. Bubbling the solutions with oxygen prior to irradiation enhances the superoxide spin adduct signal and the inclusion of superoxide dismutase in the mixture suppresses this spin adduct signal. These observations show that the irradiation of protoporphyrin results in the production of superoxide.

4. Discussion

These results suggest that singlet oxygen may not be the only agent responsible for the photodamage in erythropoietic protoporphyria. We have shown that when superoxide is generated in the presence of trace amounts (10^{-5}–10^{-6} M) of iron, ·OH, perhaps the most potent oxidizing agent that may arise in a biological system, is formed [10,11]. Using different experimental approaches other workers have also observed the iron-mediated formation of ·OH [12–14]. These observations are consistent with the following mechanism:

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + O_2^- \longrightarrow Fe^{3+} + H_2O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow {\cdot}OH + OH^- + Fe^{3+}$$

O_2^- has been shown [15] to easily diffuse through cell membranes as well as some distance in an aqueous environment. Thus, the site of ·OH radical formation may be some distance from the site of O_2^- generation provided superoxide dismutase is not present in significant amounts. The lack of superoxide dismutase outside the cell as well as the exceedingly small amounts of superoxide dismutase in the plasma [16] suggest that superoxide formation by protoporphyrin may lead to the formation of significant amounts of hydroxyl radical. Even though the yield of singlet oxygen is undoubtedly much higher than that of hydroxyl radical, the extreme reactivity of hydroxyl radical compared to singlet oxygen suggests it may be responsible for some of the damage that occurs in protoporphyria. For example, the reaction rate constant of methionine with ·OH in aqueous solution (pH 7.0) is 8.5×10^9 M^{-1} s^{-1} [17], while it is only 3×10^7 M^{-1} s^{-1} for singlet oxygen in CH_3OH solution [18]. Similarly, the rate constant for reaction of ·OH with benzene is 7.8×10^9 M^{-1} s^{-1} [19], while it is only 3×10^3 M^{-1} s^{-1} for singlet oxygen [20]. Thus, in considering the identity of the toxic species, reactivity as well as yield must be considered.

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